academicJournals

Vol. 6(4), pp. 145-152, April 2014
DOI: 10.5897/JENE2014.0437
ISSN 2006-9847 ©2014 Academic Journals
http://www.academicjournals.org/JENE

Journal of Ecology and the Natural Environment

Full Length Research Paper

Molecular diversity of arbuscular mycorrhizal fungi (AMF) in Lake Victoria Basin of Kenya

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Accepted 14 February, 2014

Arbuscular mycorrhizal fungi (AMF) play a key role in land reclamation, sustaining soil fertility and cycling of nutrients, which in turn increases plant vigour and productivity. AMF differ in both structural characteristics and global distribution, which is strongly correlated with the respective functional role. This study investigated the molecular diversity of arbuscular mycorrhizal fungi (AMF) in selected representative farmlands across Lake Victoria Basin and wheat farms in Njoro District of Kenya. Native AMF genera were identified by morphological techniques and their molecular diversity assessed by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) techniques and genetic distance analysis. In all five field sites, three AMF genera were identified with varying relative abundances, namely, *Glomus* (50%), *Scutellospora* (30%) and *Gigaspora* (16%). Lambwe fields had the highest spore densities (13 spores per gram dry weight) and evenness (0.84) while Kibos and Njoro had least spore count (4 - ditto) and evenness (0.32), respectively. The AMF population from Njoro wheat farms had highest heterozygosity (He = 0.257) and hence was the most genetically diverse compared to other populations.

Key words: *Glomus* spp., *Gigaspora* spp., *Scutellospora* spp., molecular diversity.

INTRODUCTION

Productivity of agricultural land among small-holder peasant communities is diminishing due to depleted soil fertility and destabilized nutrient acquisition by plants. Major factors that constrain tropical soil fertility and sustainable agriculture are low nutrient capital, moisture stress, erosion, increased phosphorus fixation, high acidity with aluminium toxicity, and low soil biodiversity. The fragility of many tropical soils limits food production in annual cropping systems (Bonfante and Perotto, 2000; Ashman and Puri, 2002; Muchovej, 2004).

Arbuscular mycorrhizal fungi (AMF) play a key role in land reclamation, sustaining soil fertility and cycling of nutrients, which in turn increases plant vigour and productivity. Over 80% of plant species are associated with mycorrhizal fungi, 67% of which is AMF. AMF differ in both structural characteristics and global distribution, which is strongly correlated with the respective functional role. Soils typically contain several species of AMF, a combination of which is needed to function as an adequate plant-soil interface (Wardle and Van der Putten, 2002). Mycorrhizal fungi also play a significant role in the regulation of soil biological activity because of their abundance throughout the uppermost soil layer. The external mycelium of AMF acts as an extension of host plant roots and serves as a direct link between roots and soil nutrient reserves. The mycorrhizal fungal hyphae are involved with the scavenging and retention of nutrient ions, and with the creation of an aggregate system that

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acts as a control point for accrual and mineralization of organic matter in the soil, which creates a system that reduces erosion and leaching loss of nutrients (Gianinazzi and Gianinazzi-Pearson, 1986; Finlay and Soderstrom, 1992).

Such mycorrhizal associations also helps in maintenance and improvement of soil structure, the uptake of relatively immobile elements, both macronutrients and micro-nutrients, the alleviation of aluminium and manganese toxicity, the interactions with other beneficial soil organisms, and improved protection against pathogens (Harley and Smith, 1983; Xavier and Germida, 1998; Buking and Sachar-Hill, 2005; Cardoso and Kuyper, 2006). The mutual symbiosis between AMF and plants is known to increase plant vigour and productivity, especially under unfavourable conditions (Lendzemo and Kuyper, 2001). This could become a useful biotic interaction for effective and sustainable soil management and reclamation systems for improved productivity. The aim of this study was to investigate the molecular diversity of AMF in selected sorghum farmlands in Lake Victoria Basin and wheat farms in Njoro District of Kenya.

MATERIALS AND METHODS

Physicochemical characteristics of mycorrhizal soils

Five sampling sites were selected namely, Alupe, Kibos, Lambwe, and Oyugis in Western Kenya, and Njoro wheat fields in the Rift Valley region of Kenya. Soil samples were taken from homogeneous areas in terms of landscape and crop age at each site, by collecting 10 single samples consisting of 0.5 L of soil and roots of each plant. Each single sample was collected within the plant root area and 5 to 10 cm deep. The single samples per field site were pooled to make one compound sample and analyzed for physicochemical characteristics: pH, soil type, soil texture, soil drainage, at Egerton University. The sampling was done in the dry seasons (January and February) for two consecutive years.

Isolation of arbuscular mycorrhizal fungi (AMF)

Pure isolates of AMF: *Glomus etunicatum, Scutellospora fulgida*, and *Gigaspora margarita*, were obtained from cultures kept and Mycorhiza research laboratory at National Museums of Kenya. AMF spores were isolated by wet-sieving and decanting density-gradient centrifugation method as described by Schenk and Perez (1990). One hundred grams of soil sample was placed in a 2.0 L container and vigorously mixed with 1.5 L of water to free spores from soil and roots. The suspension was left to settle for 45 min, decanted and the supernatant sieved using a 25 µm mesh sieve over a 45 µm mesh sieve. The sievings were transferred to 50 mL centrifuge tubes with a fine stream of water from wash bottle and centrifuged at 1300 × g in a swinging bucket rotor for 3 min. The supernatant and adhering organic debris were removed carefully and the soil pellet suspended in chilled 1.7M sucrose then centrifuged at 1300 × g for 1.5 min. The supernatant was poured through the 25 µm mesh sieve and rinsed with tap water. The spores were washed into a Petri dish and sorted into morphotypes and counted using a dissecting microscope at a magnification of × 50.

Samples (0.5 g) of plant roots collected from the field were placed in perforated plastic holders (OmniSet tissue cassette; Fischer Scientific, Pittsburg, PA.) and stored in cold water until they were processed. Samples were covered with 200 mL of 1.8 M KOH in a beaker and heated to 80°C in a fume hood for 30 min, then rinsed with water and briefly with dilute hydrochloric acid solution (5.0 mL conc. HCl in 200 mL H_2O), stirred and drained. Lactoglycerol trypan blue stain was dispensed into a beaker and heated to 80°C. Samples were placed in the stain for at least 30 min, then destained with lactoglycerol followed by two changes of tap water. The cleared and stained roots were spread in a scribed 10 cm diameter Petri dish and observed under dissecting microscope for root colonization (Phillips and Hayman, 1970).

Morphological Identification of AMF Isolates

Several spores from the same morphological spore type were observed under a dissecting microscope at a magnification of × 50. Spores were put in a watch glass or a small Petri dish and their shape, size, colour, hyphal attachment, auxiliary cell, sporocarp, germination shield, and surface ornamentation observed following Morton and Redecker (2001), and identified to genus. AMF spores from each 100 g soil sample were counted and data expressed as mean spore density (numbers per 100 g sample). Relative abundance of each species in each field site was calculated as:

$$\text{Relative abundance} = (n_i/N_j) \times 100$$

Where, n_i = number of spores that belong to species i and N_j = total number of spores in the site. The mean of six replicates was expressed as percent relative abundance. Significant differences were separated by Fisher's LSD test at $p<0.05$ confidence level. Mycorrhizal fungal diversity was calculated by using the Shannon index (H'), which combines two components of diversity, species richness and evenness of individuals among the species (Vestberg, 1999).

$$H' = -\Sigma P_i \ln P_i \text{ and; } E = H' / H_{max}$$

Where, H' = Shannon index, P_i = proportion of the ith species, ln = natural logarithm, E = evenness, H_{max} = Diversity maximum when all species are equally abundant.

Molecular characterization of AMF

Fungal DNA was extracted from the spores according to Lee et al. (1988) with a few modifications. Samples were vortexed for 2 min and centrifuged at 13000 rpm for 5 min and the supernatant decanted. Three freeze thaw cycles were performed with liquid nitrogen and samples were crushed in 5.0 mL of 2% Cetyltrimethylammonium bromide (CTAB) buffer with the aid of a sterile micro pestle to ensure effective lysis of cells. Proteinase K (20.0 µl, 2.0 mg/ml) was added and allowed to stand at room temperature for 15 min. Samples were incubated at 65°C for 45 min with intermittent vortexing every 15 min. Chloroform (400 µl) was added after incubation, vortexed and centrifuged at 13000 rpm for 5 min. The aqueous phase was further extracted with another 0.5 mL of phenol: chloroform: isoamyl alcohol (24:24:1 by volume). 0.45 mL of the aqueous phase were mixed with 0.045 mL of 5.0 M ammonium acetate and 0.9 mL of cold ethanol and incubated at -20°C. After 1 to 2 h, precipitated DNA was pelleted by centrifugation at 11,800 × g for 15 min at 4°C, washed with cold 70% ethanol, dissolved in 50 µl of deionized water and incubated with 5.0 µl (50 µg) of GIBCO BRL RNase T1 for 30 min at 37°C. After extraction, DNA concentration was estimated on 0.8% agarose

Table 1. Characteristics of soils collected from the five field sites in Kenya.

Zone /Site	Altitude (m)	Latitude / longitude	pH	Soil characteristic
Alupe	1189	00°29'N / 34°08'E	6.3	Ferro-orthic acrisols, sandy clay; well drained
Kibos	1214	00°04'S / 34°48'E	6.1	Alluvial, vetroeutic planosol, sandy loam; Well drained
Lambwe	1440	00°31'S / 34°22'E	7.1	Vertisols of clay texture; well drained.
Njoro	2164	00°19'S / 35°56'E	6.6	Haplic, verto-luvic phaeozems; well drained
Oyugis	1320	00°30'S / 34°44'E	6.4	Chromo-luvic phaeozem; well drained

gels buffered in 1xTBE (89 mM tris-HCl (pH 8), 89 mM boric acid and 2.0 mM EDTA) in a horizontal electrophoresis apparatus. Before loading into the gels, 10.0 µl of the DNA samples were mixed with 5.0 µL of 1 × gel loading buffer III (0.25 % bromophenol blue, 0.25% xylene cyanol and 30% glycerol). Standards of 2.5, 5.0, 10.0 and 20.0 ng of uncut unmethylated λ-DNA were loaded onto the gel and the gels run at 150V for 30 min. Gels were imaged with the gel documentation system. DNA content was estimated by comparing resultant DNA bands with those of the standards. Solid bands with no streaks signified high molecular weight DNA. After the estimation of DNA, individual extractions were adjusted with TE buffer to a standard concentration of 10.0 ng/µL and stored at -20°C (Miyumi et al., 2004).

Detection of inter- and intra-specific differences among the AMF isolates was analyzed by RAPD-PCR (Random amplified polymorphic DNA polymerase chain reaction) (Lee et al., 1988). Dilutions of genomic DNA were prepared in sterile distilled water, such that the final DNA concentration was 2.5 ng/µL. These dilutions were stored at -20°C and were only thawed twice before discarding. Amplification of DNA fragments was carried out by PCR using 10-mer arbitrary primers (Operon Technologies Inc., California, USA). The PCR conditions for each of the 18 RAPD markers were optimized and PCR reactions were set up in 10.0 µL volumes in 96-well PCR plates (Perkin Elmer, Germany). The amplification reaction was carried out in a 0.2 mL tubes using a programmable thermal cycler (Corbett Research, Germany). Each PCR reaction mixture contained 1.0 µL (2 pmol) of RAPD primer, 1.0 µL of 10 × PCR buffer, 1.0 µL of 50 mM $MgCl_2$, 0.5 µL (0.2 mM) dNTPs, 0.15 µL (0.2 U) Taq polymerase, 5.85 µL SDW and 0.5 µL (1.0 ng) of DNA in a final volume of 10.0 µL.

The reaction mixture was denatured for one minute at 94°C, annealing for one minute at 36°C, and extension for 5 min at 72°C with a final extension for 2 min at 72°C. A negative control (master mix without template DNA) was incorporated in every PCR run. The reproducibility of RAPDs was tested by repeating a subset of samples across PCR runs. PCR products were resolved by electrophoresis on 1.5% agarose gel in 1 x TBE buffer by running at 150 V for 3 h. Four microlitres of 1 x loading buffer was added to the PCR products and then were analyzed by electrophoresis on agarose gel in 0.5 × TBE (tris borate EDTA) containing 0.5 µg of ethidium bromide per mL. A 100 bp DNA ladder (Invitrogen, USA) was used as a molecular weight marker. Gels were imaged with the gel documentation system (BioRad GelDoc 2000 Documentation System).

Genetic diversity analysis

The bands on agarose gel were scored on a spreadsheet as '1' for the presence and '0' for the absence of DNA bands for each isolate. Bands of the same size (assessed by eye) in each PCR run were assumed to be homologous. The following genetic diversity parameters: polymorphic RAPD fragment (for convenience, each was treated as an allele) frequencies, effective number of alleles, Shannon's information index, proportion of polymorphic loci and, expected heterozygosity were computed for each AMF population using GENALEX v6.41 software (Peakall and Smouse, 2006). The same software was used to calculate genetic distance and identity based on Nei's index (Nei, 1972). A dendrogram was constructed based on Nei's genetic distances using PowerMarker v3.0 software (Liu, 2005) and viewed in TreeView software.

RESULTS AND DISCUSSION

Physicochemical characteristics of mycorrhizal soils

The mycorrhizal soils collected from the various field sites showed different physicochemical characteristics (Table 1). All the field sites were well drained but had varying soil pH, ranging from 6.1 in Kibos to 7.1 in Lambwe. Field sites ranged from altitude 1189 m (Alupe) to 2164 m (Njoro) above sea level. All field sites were well drained but varied in soil types. Soil types determine soil pore size, which in turn influences nutrient retention capacity in soils as well as the soil physical and chemical characteristics (Ashman and Puri, 2002).

Morphological identification of AMF isolates

Three AMF genera identified were: *Glomus* spp., *Scutellospora* spp., and *Gigaspora* spp. (Table 2). Significant differences in richness and relative abundances of indigenous AMF were observed in all the field sites. Lambwe site had the highest total spore count (12.59) while Kibos had the lowest (4.23). *Glomus* were dominant AMF (49.74%) in all field soils followed by *Scutellospora* (29.60%) and *Gigaspora* (15.80%). Other fungal spores were also observed but could not be conclusively identified. The high spore densities observed in Lambwe suggest that the soils have experienced less tillage over the years. This is in agreement with findings by Douds et al. (1993) who reported that AMF spore densities are generally high in low-input agricultural fields and lower at both native and high input sites (Smith and Dickson, 1997). Agricultural practices are known to affect AM fungal community structure and as such low spore densities and mycorrhization have been reported

Table 2. Relative abundance and spore counts of AMF genera sieved from 100 g of field soils, means and standard deviations based on 6 replicate counts of each sample.

AMF species	Spore count					
	Alupe	**Kibos**	**Lambwe**	**Njoro**	**Oyugis**	**Mean**
Glomus	(49.18) 3.90^{c}	(51.06) 2.17^{d}	(44.42) 5.61^{d}	(54.96) 4.54^{d}	(49.06) 2.87^{c}	(49.74 ± 1.33) 3.82 ± 0.68^{a}
Scutellospora	(32.28) 2.56^{b}	(28.71) 1.22^{c}	(30.09) 3.80^{c}	(28.21) 2.33^{c}	(28.72) 1.68^{b}	(29.60 ± 0.87) 2.32 ± 0.34^{b}
Gigaspora	(12.23) 0.97^{a}	(15.29) 0.65^{b}	(19.32) 2.44^{b}	(12.83) 1.06^{b}	(19.32) 1.13^{b}	(15.80 ± 0.43) 1.25 ± 0.19^{c}
Others (unidentified)	(6.31) 0.50^{a}	(4.94) 0.21^{a}	(6.18) 0.78^{a}	(3.99) 0.33^{a}	(2.91) 0.17^{a}	(4.87 ± 0.21) 0.40 ± 0.02^{d}
Total	**7.93 ± 1.02**	**4.23 ± 0.08**	**12.59 ± 1.58**	**8.20 ± 1.23**	**5.85 ± 1.01**	**7.79 ± 1.41**

Values are mean ±SE; Values in brackets are percent abundances; Letters show vertical comparisons among treatments at P = 0.05 error level; Means with the same letter are not significantly different from each other.

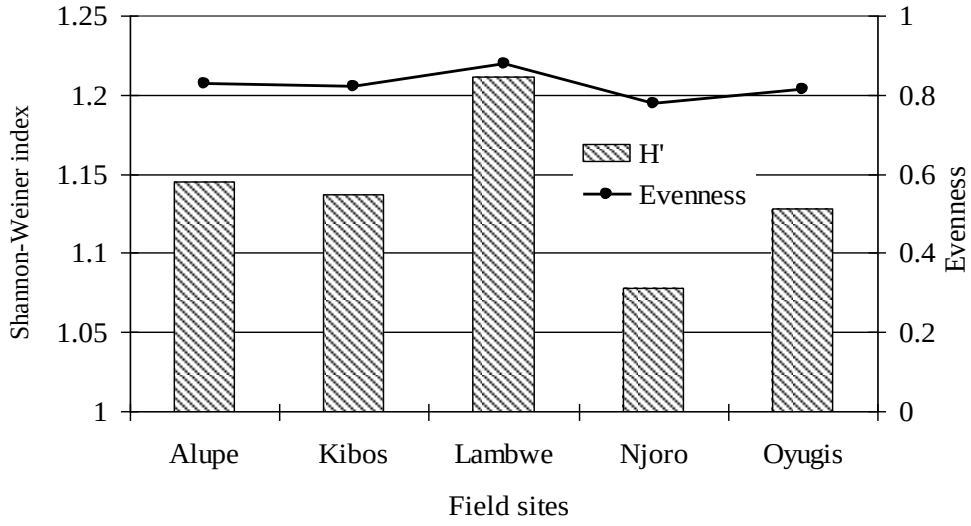

Figure 1. AMF species diversity as measured by the Shannon index (H').

(Douds and Millner, 1999), hence the high variability in spore density among the field sites. Furthermore, presence of non-sporulating species may not be detected through the standard technique (Schenck, 1982; Liu and Luo, 1994).

The species diversity measured by Shannon-Wiener diversity index (H) differed significantly between field sites (Figure 1). Lambwe field soils showed a higher degree of AMF diversity (H = 1.21) while Njoro had the least (H = 1.08). The possible reasons for observed biodiversity index and species abundance were the complex ecological system structure and high plant species diversity. Evenness of species population did not show significant variation across field sites except at Lambwe (E = 0.88) which was significantly high (Figure 1). This means the spatial heterogeneity of different field sites had significant influence on AMF genetic diversity. Such influence together with seasonal variations were also detected by Kwong-ma (2004) who reported that species of AMF (*Acaulospora colossica*) sporulate at the beginning of summer, remain viable as spores throughout the summer period and is only physiologically active in the cool season plant (for example, wild garlic) community. Similarly, de Oliveira and de Oliveira (2005) concluded that AMF sporulation is seasonal, dependent on soil moisture and other soil factors. There is a tendency that indigenous species of AMF though not active at one seasonal period, may become effective pending favourable environmental conditions that aids colonisation and subsequent release of spores into the soil.

Molecular characterization of AMF Isolates

A set of reproducible bands was produced from amplification of AMF DNA fragments using a particular primer and was defined as a “pattern”. Out of the 18 arbitrar

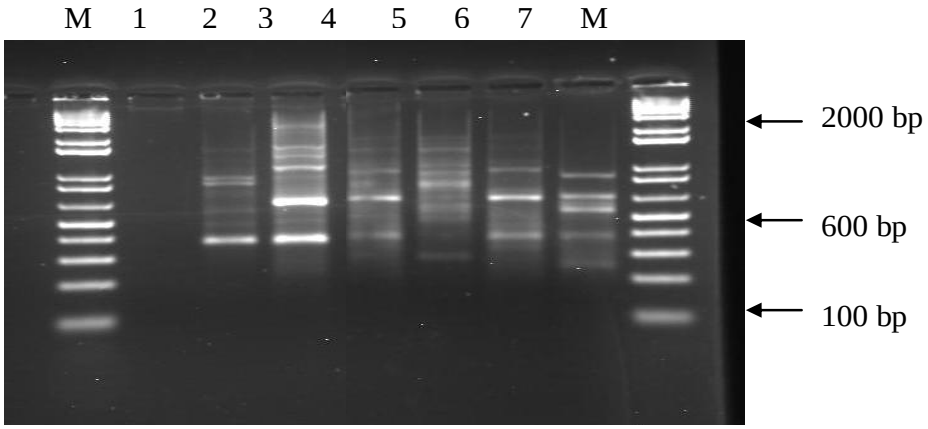

Figure 2. Representative Ethidium bromide stained agarose gel containing RAPD-PCR products of *Glomus* spp. isolate amplified with arbitrary primer OPB-11. Lanes M are 100-bp DNA ladder, 1-negative, 2-Alupe, 3-Kibos, 4-Lambwe, 5-Njoro, 6-Oyugis isolates, 7-positive (*Glomus etunicatum*).

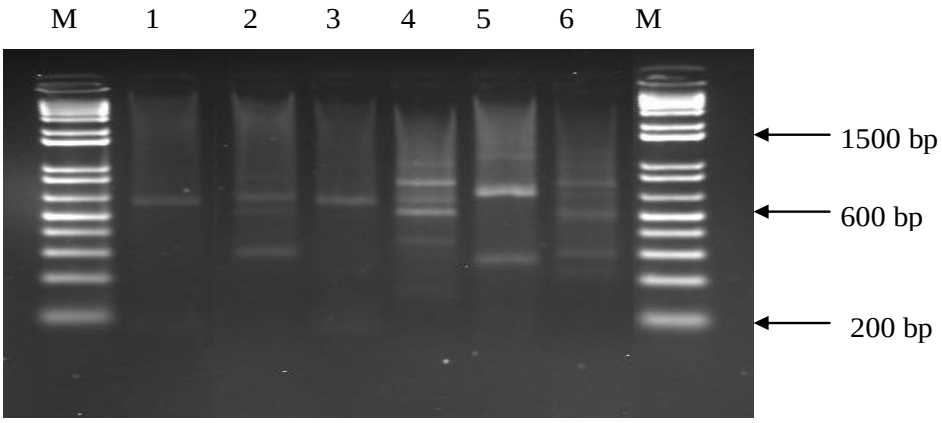

Figure 3. Representative Ethidium bromide-stained agarose gel containing RAPD-PCR products of *Scutellospora* spp. isolate amplified with arbitrary primer OPB-11. Lanes M are 100-bp DNA ladder, 1-Alupe, 2-Kibos, 3-Lambwe, 4-Njoro, 5-Oyugis isolates, 6-positive (*Scutellospora fulgida*).

primers screened, 4 primers namely, OPB-11 (5'-GTAGACCCGT-3'), OPE-07 (5'-AGATGCAGCC-3'), OPE-11 (5'-GAGTCTCAGG-3') and OPA-02 (5'-TGCCGAGCTG-3'), were selected because they gave clear polymorphism (Figures 2 to 4). Primer OPB-11 produced 5 amplicons and the sizes of RAPD-PCR products ranged between 200 to 1800 bp. It was noted that the majority of RAPD-PCR primers gave distinctly reproducible bands in all the five field populations. *Glomus* spp. from various field sites had common bands with 500 and 1000 bp, except Njoro population which had unique bands with 300 bp and 1200 bp. *Scutellospora* spp. had common bands with 800 bp in all samples while Alupe and Lambwe population lacked a common band with 350 bp (Figure 2). Positive control (*Scutellospora fulgida*) had 6 clear bands compared to other populations which had less (Figure 3). *Gigaspora* spp. had common bands with 800 bp except Njoro population which had unique band with 400 bp. The results obtained showed genetic differences within populations across field sites. The genetic variability observed across populations as is often seen with RAPD-PCR (Caldero et al., 2004).

Genetic diversity analysis

The percentage of polymorphic loci was variable ranging from 38.46% in Lambwe to 61.54% in Njoro and Oyugis

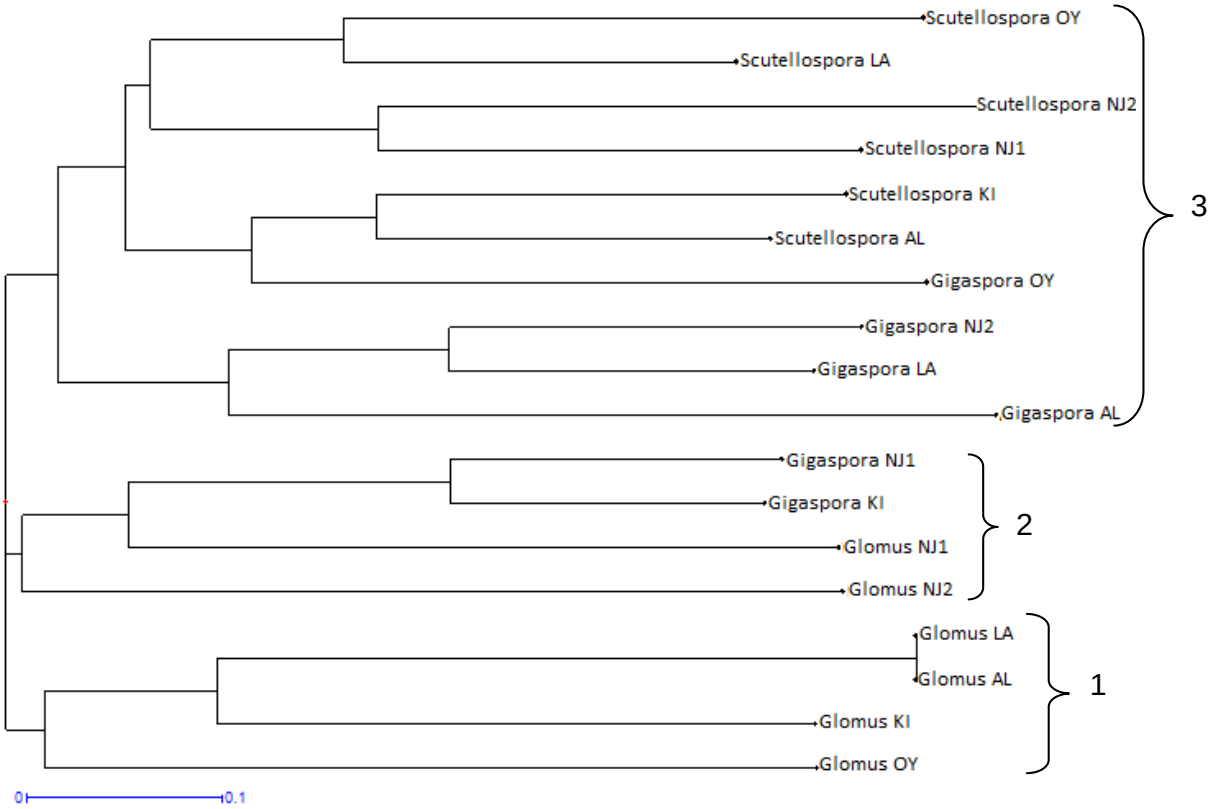

Figure 4. Dendrogram based on Nei's genetic distance. AMF sources: AL = Alupe, KI = Kibos, LA = Lambwe, NJ1 = Njoro, NJ2 = Positive control, OY = Oyugis.

Table 3. Analysis of polymorphism obtained with RAPD primers in different populations of AMF spp.

Population of AMF spp.	Percentage of polymorphic loci	Shannon information index, I	Mean Heterozygosity, He
Alupe	53.85 ± 2.79	0.288 ± 0.079	0.190 ± 0.054
Kibos	46.15 ± 2.51	0.251 ± 0.081	0.167 ± 0.055
Lambwe	38.46 ± 1.97	0.215 ± 0.080	0.144 ± 0.055
Njoro	61.54 ± 4.01	0.372 ± 0.088	0.257 ± 0.062
Oyugis	46.15 ± 2.33	0.283 ± 0.090	0.196 ± 0.064
Control	61.54 ± 4.23	0.356 ± 0.085	0.242 ± 0.059
Mean ±SE	**51.28 ± 3.80**	**0.294 ± 0.034**	**0.200 ± 0.023**

Values are mean ±SE

populations and the overall mean was 51.28% (Table 3). The high percentage of polymorphic loci observed reflected noticeable levels of genetic polymorphism in the studied populations. Njoro population gave the highest Shannon information index (I = 0.372) while Lambwe population had the lowest (I = 0.215) with an overall mean of 0.294 (Table 3). The Shannon information index was clearly different from zero and reflects presence of genetic diversity. The mean expected heterozygosity among individual populations varied from 0.144 at Lambwe to 0.257 at Njoro and the overall mean was 0.200. The Njoro population was most genetically diverse (He = 0.257) than other populations (Table 3).

The genetic distance between the population ranged from 0.003 to 0.182 while the genetic identity ranged from 0.833 to 0.997 (Table 4). The highest genetic similarity

Table 4. Pairwise population matrix of Nei's unbiased measures of genetic distance (below diagonal) and genetic identity (above diagonal) for AMF spp.

Population of AMF spp.	Alupe	Kibos	Lambwe	Njoro	Oyugis	Control
Alupe	***	0.959	0.974	0.935	0.997	0.908
Kibos	0.042	***	0.995	0.932	0.990	0.865
Lambwe	0.027	0.005	***	0.878	0.985	0.833
Njoro	0.068	0.075	0.130	***	0.996	0.958
Oyugis	0.003	0.010	0.015	0.004	***	0.954
Control	0.096	0.145	0.182	0.042	0.047	***

was found between Oyugis and Alupe populations (0.997) while the lowest was resolute between Lambwe populations and positive control (0.833). The Nei's genetic distance was used to construct phylogenetic tree using PowerMarker software. Three major clusters labeled 1 - 3 were observed. Within each cluster were subclusters which suggest intrageneric differences across all the populations. Cluster 1 corresponded to Glomerales since they consisted of only *Glomus* spp. from different populations. Clusters 2 and 3 corresponded to Diversisporales since they contained both *Scutellospora* and *Gigaspora* spp. (Figure 4). The *Glomus* species in cluster 2 was likely *Glomus* group C which is a basal member of the Diversisporales (Redecker and Raab, 2006). The dendrogram also suggested that AMF undergoes major part of genetic variation by environmental factors. It is also possible that the lack of large differences among the various glomalean populations cover a variation in functional adaptability of identical genotypes. This would lead to differences only at an epigenetic level, for example, by regulation of gene expression (Stukenbrock and Rosendahl, 2005). The observed diversity could be evidence for genetic redundancy or an adaptive mechanism that allows symbiosis with different plants in a whole range of environments as well as fungal stress response whereby fungal ecotypes are better adapted to specific soil types.

The simple morphology of the spores apparently has concealed the large genetic variation within the polyphyletic genera. These findings also indicated that soil chemistry and geographic factors may have a stronger influence on population structuring in agriculturally used soils than previously recognized. Other soil characteristics, such as other microorganisms, might be responsible for the observed results. These microbes are likely to interact with AMF in different ways which can lead to different genetic structures of populations (Vandenkoornhuyse et al., 2001). Moreover, new primers which were not accessible for our research, have been developed that are more specific to the Glomeromycota and would provide better coverage across the Glomeromycota and also offer the possibility of a more reliable phylogenetic placement of the environmental AMF sequences (Lee et al., 2008).

Conclusion

Three genera of AMF were identified in decreasing abundance: *Glomus, Scutellospora* and *Gigaspora* spp. *Glomus* was the dominant genus throughout all populations and could be a candidate for screening high ecological restoration strains for these areas. Lambwe populations gave highest AMF spore densities while Njoro populations were most genetically diverse. The dendrogram clustering further confirmed the molecular heterogeneity of AMF in Lake Victoria basin.

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